animals

MDPI

Communication

Distress regulates different pathways in the brain of common carp: an initial study

Alexander Burren[1], Constanze Pietsch[1]

[1] Hochschule für Agrar-, Forst- und Lebensmittelwissenschaften (HAFL), Applied University Berne (BFH), 3052 Zollikofen, Switzerland; alexander.burren@bfh.ch

* Correspondence: constanze.pietsch@bfh.ch

Received: date; Accepted: date; Published: date

Simple Summary: The aquaculture sector provides for nearly half the world's seafood consumption, thanks to its large expansion over the last 30 years. Despite this intense growth, clear guidelines for responsible practices and animal wellbeing are lacking. Gene expression studies are a fundamental tool for understanding welfare, but stress-markers in aquaculture fish species are poorly studied. In addition, the biostatistical analyses of gene expression data is not trivial and the present study applies different statistical methods in order to evaluate potential differences in the gene expression levels between control fish and fish acutely stressed by air exposure.

Abstract: For the present study, a stress trial with common carp, which is one of the most important species in aquaculture world-wide, was conducted to identify relevant gene regulation pathways in different areas of the brain. Acute distress by air exposure significantly activated the expression of the immediate early gene *c-fos* in the telencephalon. In addition, evidence for regulation of the two *crf* genes in relation to their binding protein (*crh-bp*) have been highlighted in this initial study. Inference about the effects of distress by air exposure has been obtained by using point estimation which allows the prediction of a single value that is the best description of the up to know mostly unknown effects of stress in different brain regions of carp. Furthermore, principal component analyses have been performed to reveal possible regulation patterns in the different parts of the fish brain. In conclusion, these initial studies on gene regulation in the carp brain influenced by exposure to a stressor reveal that a number of genes may be successfully used as markers for exposure to unfavourable conditions.

Keywords: aquaculture; stressors; carp; early immediate genes; biostatistics

1. Introduction

Distress is defined as a condition that interferes with the well-being of an animal if adaptation processes of the organism fail to return the physiological and/or psychological homeostasis of the animal [1,2]. Fish in aquaculture are often subjected to distress for short periods (acute stress), but can also be exposed to stressors for longer time periods (chronic stress), meaning that the body's biological functions are sufficiently altered and its coping mechanisms overwhelmed [1].

Understanding the immediate effects of stressors, such as handling and crowding, on gene regulation in fish has been a focus of aquaculture research [3–5] , in the interest of improving survival, growth, reproduction and fillet quality. The brain however, despite its role as first actor in the stress-cascade, has comparatively received little attention. Recently, a study in European seabass (*Dicentrarchus labrax L.*) and gilthead seabream (*Sparus aurata L.*) showed not only relevant differences between species, but the importance of studying the different fish brain areas separately [6]. In

addition, even certain brain areas may show significant differences in gene expression [7]. The main purpose of the present work is to identify stress-related biomarkers in different brain parts of common carp (*Cyprinus carpio*) to allow a precise evaluation of their rearing conditions.

The diversity of main functions between the regions of a fish brain have been already studied in the past. The telencephalon, for instance, has long been regarded solely with olfactory functions [8], but more recent research has confirmed its important role in the expression of emotional and motivational behaviour, as well as in fear conditioning in teleosts, including a pivotal role of these behaviours being attributed to the amygdala [9–11]. From mammals, it is known that the amygdala plays an essential role in mediating negative and positive emotions, which also involves the appraisal of incoming signals [12–15]. The optic tectum, directly connecting the efferent neurons with incoming retinal fibers in teleosts, has been recognized as essential for visually mediated behaviours already in early times [16,17]. However, the optic tectum is not solely required for perception of motions, but has more recently been proven to be essential for the correct pacing of saccades during optokinetik responses in zebrafish [18]. In addition, the hypothalamus plays an important role in energy homeostasis and appetite regulation. The activated or suppressed neurons then lead to adjustments in behaviour and metabolism. Proopiomelanocortin (*pomc*) neurons appear to drive satiety in the hypothalamic arcuate nucleus of mice [19]. The cerebellum of fish is responsible for the coordination of body movements [20], and has been linked to spatial navigation [21].

The effects of stressors on the brain can be assessed by analysing the activities of different gene sets. Firstly, brain activity can be assessed, for instance, by measuring the increased expression of stress-related immediate early genes (IEGs). For instance, *c-fos* is widely used as a functional marker of neuronal activity after a diversity of stimuli in vertebrates, due to its very rapid and robust expression [22]. In fish, different stimuli have been shown to induce varying levels of *c-fos* expression. Light avoidance as an innate choice behaviour involves rapid changes of the expression of *c-fos* in the medial zone of the dorsal telencephalon in adult zebrafish, *Danio rerio* [11]. In addition, the administration of D-amphetamine, known to activate the reward system, resulted in increased expression of *c-fos* in the same brain region 30 min after the injection of the substance [9]. Moreover, the sleep and wake behaviour of zebrafish also leads to typical differences in *c-fos* patterns in zebrafish [23]. Even caffeine has been proven to act as a stimulator of *c-fos* in the zebrafish brain [24]. In addition, exposure to neurotoxins for 60 min resulted to rapid changes of the *c-fos* protein in different brain parts of killifish, *Fundulus heteroclitus* [25].

Another gene belonging to the group of the IEGs is *egr-1* (encoding for the early growth response protein 1) which has already been shown to be changing during the breeding cycle of sticklebacks [26]. Furthermore, the detection of phosphorylated extracellular signal–regulated kinase (*erk*) by immunohistochemistry has also been used as a readout of neural activity in fish at a whole-brain level [27]. The protein palladin (*palld*) is essential for the organization of the actin cytoskeleton and a deficiency can lead to a failure of neurite outgrowth in rats [28]. Interestingly, in mirror carp (*Cyprinus carpio*) exposed to koi herpes virus, *palld* has also been described as an immune-related gene [29]. The importance of this protein in cytoskeleton organization and kidney function has been confirmed in zebrafish [30,31]. Since it may also play an important role in the organization of the fish brain, this gene was included in the present study. In addition, one metabolic gene (*gadph*) was included in the present study, since its activity in tissues implicates higher energy demands and therefore increases of the metabolic gene expression.

Secondly, the response to stressors also commonly involves activation of genes of the hypothalamus-pituitary-interrenal (HPI) axis [32] and a number of these genes has therefore also

been included in the present study. *Crf* and its receptors play an important role in the stress signalling via the HPI axis [33]. The abundance of the *crh*-binding protein (*crh-bp*) determines the availability of *crh* to its receptors, although also other biological functions of *crh-bp* have been proposed [34]. Finally, the release of stress hormones such as cortisol and 11-deoxycorticosterone lead to the activation of glucocorticoid receptor (*gr*)- and mineralocorticoid receptor (*mr*)-mediated signaling pathways in teleosts [35]. In carp, *gr2* is the most sensitive corticoid receptor, followed by the *mr* and *gr1a* and *gr1b* [36].

Thirdly, other brain networks are also known to be involved and/or affected by stress responses in other fish species. As one example, the serotonergic pathways are affected by stress in trout [37]. Serotonin also plays a role in the habituation to startling acoustic stimuli in zebrafish [38]. While serotonin agonists have anxiolytic effects in humans [39], chemicals including ethanol have shown that the acute anxiolytic effects by these substances are likely mediated by γ-aminobutyric acid receptors A (*gabaa*, [40]). The same psychoactive compounds have also been able to influence the normal behaviour of zebrafish [41]. In addition, isotocin is, together with vasotocin, a neurotransmitter and neuromodulator that is produced in distinct neurosecretory neurons in the hypothalamic nuclei [42]. Both influence the result of different behaviours, the establishment of the social status, but are also involved in osmoregulation and stress responses in fish [42]. Osmoregulation is also regulated by prolactin in fish [43]. In addition, early development, behaviour, growth, and immunoregulation depend on prolactin and prolactin receptor expression [30,44]. In neuronal tissue, prolactin is also involved in the activation of neurons that evoke action potentials and/or calcium influx in neurons [45]. These reactions culminate in the release of neurotransmitters, e.g. dopamine in the hypothalamus of rats, being able to exert a negative feedback on the prolactin release [46–48]. Interestingly, an inhibition of the prolactin release from the pituitary of trout by GABA mediated probably by both, GABA receptors A and B has been described by Prunet et al. [49]. However, the influence of stressors on these brain regulation pathways in other fish species is mostly unknown, which is the reason why a wider range of genes was included for the present study.

The present study was conducted to yield initial data on the differential gene expression patterns in the carp brain of stressed fish compared with non-stressed animals, as controls, and to apply different biostatistical methods that allow the identification of a set of potential genes suitable as biomarkers of distress in fish.

2. Materials and Methods

2.1. Rearing conditions and sampling

The fish were reared at 23 – 24°C in a 290 L aquarium equipped with a settler and a moving-bed biofilter. The carp (*Cyprinus carpio*) were kept for two months and fed 4 times daily at a feeding rate of 2 to 3 % body weight per day. During the experiment, the mean weight of fish was 28.3 g and the mean standard length was 8.9 cm. For stress treatment, fish were exposed to the air for 1 min in a net, returned to the tank and anaesthetised 30 min after that. After the acclimatization period, the control fish were taken directly from the rearing tank. Anaesthesia was performed with an overdose of tricaine methanesulfonate (MS-222, Sigma, Switzerland). The brains were sampled and stored in RNA*later*® (Sigma-Aldrich, Buchs, Switzerland) for at least 24 h and afterwards divided into the 4 brain areas (tel = telencephalon, hyp = hypothalamus, opt = optic tectum, rho = rhombencephalon comprising the corpus cerebelli and the medulla oblongata). All experimental procedures have been approved under permission number ZH-062-17 by the according Cantonal veterinarian authorities of Zurich (Switzerland).

133
134 *2.1. PCR conditions*
135 Gene expression studies have been performed by means of qPCR on a LC480 Light Cycler II
136 from Roche (Basel, Switzerland). The total RNA from each of the four parts of each brain tel, hyp,
137 opt, and rho has been extracted using RNeasy Micro Kits (Qiagen AG, Hombrechtikon, Switzerland).
138 The RNA content was confirmed using the spectrophotometer Q5000 (Quawell, San Jose, USA).
139 Subsequently, 20 µl of total RNA were reverse transcribed into cDNA using a High-Capacity cDNA
140 Reverse Transcription Kit (Applied Biosystems, distributed by Thermo Fisher Scientific, Basel,
141 Switzerland) according to the manufacturer's instructions. Thereafter, the cDNA content was
142 adjusted to 50 ng per µl using nuclease-free water (Ambion®, distributed by Thermo Fisher Scientific,
143 Switzerland) and used for real-time PCR using the LightCycler® SYBR® Green I Master mix (Roche,
144 Switzerland). All primer pairs that were used are shown in the Table S1 in the Supplement. Prior to
145 the PCR runs all primer reads have been validated, the respective PCR products confirmed by Sanger
146 sequencing and the optimal reference genes have been extracted from a set of 8 possible reference
147 genes by using the three genes with the best value for the expression stability M (i.e. *eIF4E*, *bactin*,
148 and *ef*, for more details see Table S2 in the Supplement) extracted by geNorm function in the qbase+
149 software, version 3.0 (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com) established by
150 Vandesompele et al. [51]. The target genes included early immediate genes (*c-fos*, *egr-1*, *erk-1*, *erk-2*,
151 and *palld*) as well as the metabolic gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) in order
152 to indicate active brain parts. The following genes related to the HPI axis have been included: *crf1*,
153 *crf2*, *crfr1*, *crfr2*, *crhbp*, *pomc1*, *pomc2*, *gr1*, *gr2*, and *mr*. In addition, genes of the serotonergic pathway
154 (*5-ht-r*, *serotr*) as well as *gabaa*, *iso-pre* and *prolr* have been investigated.
155
156 *2.1. Calculations and statistics*
157 As reference genes *bactin*, *eIF4E*, and *ef* have been used. The calculation of the normalized fold
158 expression of each target gene was calculated according to Taylor et al. [52]. First, the mean
159 quantitative cycle (mean ct) of the three technical replicates for each sample have been calculated.
160 The average ct of all control samples for each target gene were calculated and the relative difference
161 (Δct) between the average ct for the control group and the mean ct for each sample within each target
162 was assessed. Subsequently, relative quantities are calculated from the Δct values. For each biological
163 group (i.e. control group versus air-exposed group), a normalization factor was derived from the
164 geometric mean of the relative quantities of each reference gene. Then the relative quantity of each
165 target gene is divided by the normalization factor followed by log transformation. The obtained
166 values have then been used to calculate the geometric means for each treatment group. The standard
167 deviation (SD) and the standard error of the mean (SEM) have then been calculated from the log
168 transformed normalized expression data. The figures show the average relative normalized
169 expression for each target gene ± SEM. For the statistical calculation of the differences between the
170 means in gene expression per treatment group, non-parametric tests (Man-Whitney U tests) have
171 been run in IBM SPSS Statistics (version 26, IBM Schweiz), since it has previously been shown that
172 non-parametric calculation methods may have better control of false discovery of significant
173 differences between expression levels for example after RNA sequening [53]. Differences between
174 treatment groups were considered statistically significant when $p < 0.05$. Assuming independence of
175 tests, multiple testing leads to an inflated probability of a false positive results. To address this
176 problem the following mixed models with a fully Bayesian approach (as a part of the *brms* package

[54] in R studio, Version 1.2.1335, RStudio Team 2018) and assuming a Gaussian distribution of the data have been used to invest potential differences between the two treatment groups:

$$y_{ij} \sim N(\mu_{ij}, \sigma^2) \quad (1)$$

$$\mu_{ij} \sim \alpha_j + \beta_j x_i + \gamma_i \quad (2)$$

$$\alpha_j \sim N(0, \sigma_\alpha^2), 1, \ldots, n_{gen} \quad (3)$$

$$\beta_j \sim N(0, \sigma_\beta^2) \quad (4)$$

$$\gamma_i \sim N\left(0, \sigma_\gamma^2\right), i = 1, \ldots, n_{animal} \quad (5)$$

The models include gene specific random effects for the constants (α) and gene specific random effects for the group differences (β) and animal specific random effects for the constants (γ). The model fit has been assessed by comparison of graphical plots (QQ plots) showing the distribution of y and y_{rep}. To be better able to handle possible outliers, posteriori predictive checks based on the Markov Chain Monte Carlo (MCMC) approximation method have been applied which yielded simulated replicated data under the fitted model that have subsequently been compared to the observed data. The point estimators, their standard errors of the means, credibility intervals and posteriori p values are reported.

For an initial description of the genes mostly contributing to the common variance within the gene expression patterns in the different brain parts a principle component analysis (PCA) as a data reduction method was performed on the log transformed normalized expression data in R studio (Version 1.2.1335, RStudio Team 2018). The representation of the variables for the principle components is calculated as a cos2 value. For a given variable, the sum of the cos2 on all the principal components is equal to one.

3. Results

3.1. Immediate early genes, (*c-fos*, *egr-1*, *erk-1* and *erk-2*, *palld*) and *gapdh*

A significant difference between control fish and distressed fish was observed for the IEG *c-fos* in the telencephalon ($p<0.05$), but not in the other brain sections that have been investigated (Figure 1). In addition, an increased probability for a reduction of the expression of this gene was observed in the optic tectum in relation to other genes that have been included in the present study (Table 1 and Figure S4). However, the other IEGs have not significantly been influenced by the stress treatment

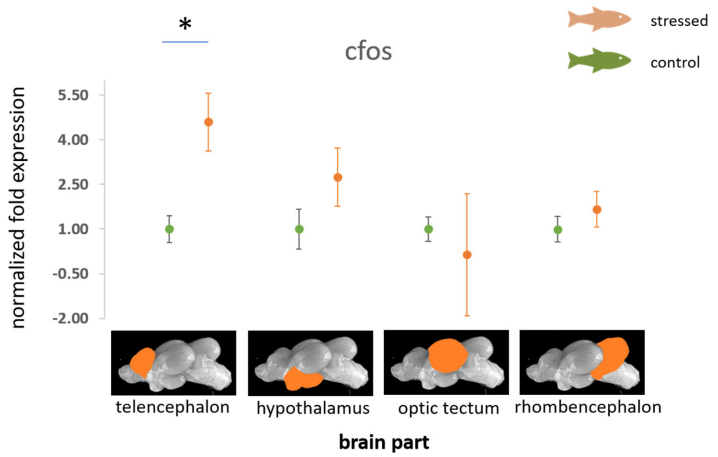


Figure 1. Gene expression profile of the immediate early gene c-fos in each of the 4 brain parts in the control fish and fish 30 min after the air exposure, mean ± SEM; n = 4 per treatment, significance according to the Mann-Whitney U tests, $p < 0.05$.

For the IEGs, the first two components extracted in the PCA for each brain part explained 77.1 % of the variance in the IEG data in the telencephalon, 83.8 % in the hypothalamus, 82.8 % in the optic tectum, and 81.1 % of the variance in the IEG data from the rhombencephalon (Figure S1 in the supplement). However, the genes that indicated a good representation on the principle component, i.e. displayed by a high cos2 value in Figure S1 (in the supplement), differed for each brain part.

Table 1. Probabilities for potential group differences between the control animals and the distressed fish (n = 4 each) in the different brain parts, the table shows for each of the genes the point estimator, the SEM in brackets, and the credibility interval and the posteriori p value in the second row.

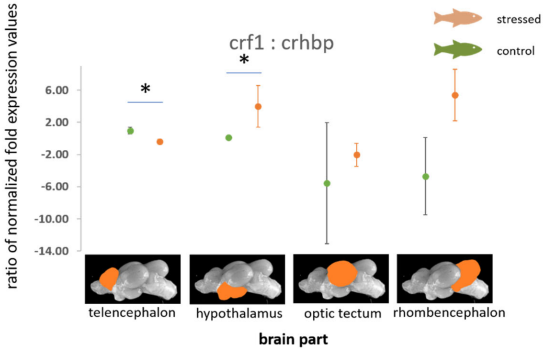
Gene	Tel	Hyp	Opt	Rho
18S RNA	**0.16 (0.59)**	**0.29 (0.46)**	**0.75 (0.83)**	**0.35 (0.46)**
	-1 – 1.39, p = 0.602	-0.5 – 1.31, p = 0.740	-0.76 – 2.47, p = 0.824	-0.52 – 1.28, p = 0.780
5-ht-r	**0.22 (0.60)**	**0 (0.41)**	**0.09 (0.82)**	**0.03 (0.45)**
	-0.9 – 1.51, p = 0.646	-0.87 – 0.86, p = 0.492	1.57 – 1.77, p = 0.540	-0.87 – 0.91, p = 0.526
eIF4E	**-0.01 (0.60)**	**-0.48 (0.51)**	**-0.66 (0.85)**	**0.03 (0.46)**
	-1.22 – 1.22, p = 0.496	-1.66 – 0.31, p = 0.162	-2.41 – 0.98, p = 0.210	-0.90 – 0.96, p = 0.520
b2m	**0.01 (0.58)**	**0.09 (0.42)**	**-0.85 (0.82)**	**0.15 (0.44)**
	-1.15 – 1.18, p = 0.515	-0.75 – 1.01, p = 0.580	-2.58 – 0.64, p = 0.144	-0.72 – 1.07, p = 0.630
bactin	**0.13 (0.60)**	**0.14 (0.43)**	**0.33 (0.82)**	**-0.08 (0.45)**
	-1.06 – 1.38, p = 0.584	-0.67 – 1.12, p = 0.621	-1.23 – 2.03, p = 0.650	-1.00 – 0.80, p = 0.432
c-fos	**0.91 (0.70)**	**0.37 (0.48)**	**-1.5 (0.93)**	**0.33 (0.46)**
	-0.26 – 2.40, p = 0.912	-0.40 – 1.49, p = 0.783	-3.41 – 0.21, p = 0.045	-0.53 – 1.28, p = 0.758
crf-1	**-0.05 (0.57)**	**0.12 (0.43)**	**-0.31 (0.82)**	**0.25 (0.47)**
	-1.26 – 1.07, p = 0.467	-0.70 – 1.08, p = 0.611	-1.97 – 1.28, p = 0.350	-0.64 – 1.22, p = 0.698
crf-2	**-0.87 (0.69)**	**0.05 (0.44)**	**1.16 (0.87)**	**0.30 (0.47)**

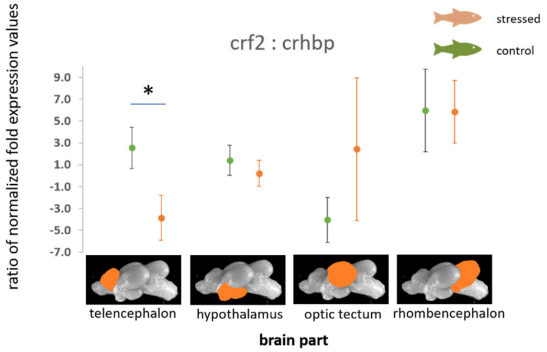
	-2.31 – 0.27, p = 0.088	-0.88 – 1.00, p = 0.546	-0.45 – 2.93, p = 0.918	-0.57 – 1.29, p = 0.734
crf-r1	**0.04 (0.57)**	**0.21 (0.44)**	**0.39 (0.80)**	**-0.37 (0.45)**
	-1.12- 1.21, p = 0.520	-0.58 – 1.20, p = 0.670	-1.17 – 2.06, p = 0.686	-1.32 – 0.47, p = 0.204
crf-r2	**-0.11 (0.58)**	**0.28 (0.46)**	**0.10 (0.81)**	**-0.31 (0.47)**
	-1.31 – 1.02, p = 0.420	-0.53 – 1.34, p = 0.727	-1.50 – 1.68, p = 0.546	-1.29 – 0.57, p = 0.260
crh-bp	**0.39 (0.61)**	**0 (0.41)**	**0.08 (0.80)**	**-0.15 (0.46)**
	-0.72 – 1.72, p = 0.737	-0.89 – 0.84, p = 0.494	-1.49 – 1.67, p = 0.545	-1.10 – 0.72, p = 0.372
ef	**-0.13 (0.59)**	**0.12 (0.43)**	**0.38 (0.81)**	**-0.1 (0.46)**
	-1.33 – 1.06, p = 0.414	-0.68 – 1.09, p = 0.606	-1.24 – 2.07, p = 0.688	-1.03 – 0.81, p = 0.411
egr-1	**-0.08 (0.59)**	**-0.04 (0.43)**	**-0.81 (0.85)**	**0.16 (0.46)**
	-1.31 – 1.07, p = 0.444	-0.95 – 0.84, p = 0.468	-2.55 – 0.76, p = 0.160	-0.77 – 1.12, p = 0.632
erk-1	**-0.15 (0.57)**	**-0.23 (0.44)**	**0.14 (0.79)**	**-0.22 (0.45)**
	-1.34 – 0.97, p = 0.395	-1.25 – 0.52, p = 0.312	-1.38 – 1.75, p = 0.567	-1.15 – 0.63, p = 0.318
erk-2	**0.10 (0.57)**	**0.27 (0.47)**	**0.65 (0.82)**	**0.02 (0.45)**
	-1.02 – 1.24, p = 0.570	-0.55 – 1.36, p = 0.717	-0.88 – 2.34, p = 0.788	-0.90 – 0.92, p = 0.513
gabaa	**-0.07 (0.58)**	**-0.08 (0.42)**	**-1.62 (0.93)**	**-0.36 (0.46)**
	-1.22 – 1.09, p = 0.454	-1.01 – 0.75, p = 0.426	-3.55 – 0.07, p = 0.035	-1.30 – 0.49, p = 0.214
gapdh	**0.09 (0.58)**	**0.41 (0.50)**	**0.53 (0.82)**	**0.04 (0.47)**
	-1.05 – 1.30, p = 0.564	-0.38 – 1.56, p = 0.807	-1.00 – 2.16, p = 0.745	-0.93 – 0.99, p = 0.539
gr1	**-0.07 (0.59)**	**-0.33 (0.46)**	**-0.90 (0.85)**	**-0.15 (0.45)**
	-1.33 – 1.04, p = 0.456	-1.37 – 0.44, p = 0.230	-2.59 – 0.68, p = 0.140	-1.06 – 0.75, p = 373
gr2	**0 (0.59)**	**0.16 (0.44)**	**0.30 (0.81)**	**-0.24 (0.45)**
	-1.21 – 1.24, p = 0.490	-0.66 – 1.12, p = 0.631	-1.29 – 1.96, p = 0.649	-1.17 – 0.65, p = 0.299
isopre	**1.21 (0.79)**	**0.12 (0.44)**	**-0.79 (0.85)**	**-0.02 (0.46)**
	-0.07 – 2.87, p = 0.953	-0.74 – 1.08, p = 0.599	2.47 – 0.80, p = 0.170	-0.07 – 2.87, p = 0.953
mr	**-0.31 (0.61)**	**-0.31 (0.47)**	**0.20 (0.82)**	**-0.25 (0.47)**
	-1.64 – 0.89, p = 0.306	-1.39 – 0.45, p = 0.256	-1.42 – 1.83, p = 0.603	-1.22 – 0.65, p = 0.298
palld	**0.11 (0.55)**	**0.20 (0.45)**	**-0.40 (0.83)**	**-0.16 (0.46)**
	-0.96 – 1.26, p = 0.577	-0.61 – 1.22, 0.656	-2.08 – 1.18, p = 0.313	-1.10 – 0.76, p = 0.363
pomc1	**0.09 (0.60)**	**-0.39 (0.50)**	**1.47 (0.93)**	**1.68 (0.73)**
	-1.08 – 1.38, p = 0.546	-1.58 – 0.39, p = 0.221	-0.22 – 3.40, p = 952	0.28 – 3.13, p = 0.997
pomc2	**0.75 (0.71)**	**-0.22 (0.44)**	**2.05 (1.12)**	**1.09 (0.63)**
	-0.41 – 2.31, p = 0.867	-1.23 – 0.56, p = 0.316	0.03 – 4.45, p = 0.977	-0.01 – 2.40, p = 0.974
prolr	**-0.45 (0.61)**	**0.14 (0.43)**	**-0.99 (0.89)**	**0.11 (0.46)**
	-1.78 – 0.66, p = 0.233	-0.67 – 1.07, p = 0.618	-2.84 – 0.63, p = 0.118	-0.77 – 1.07, p = 0.586
serotr	**-1.47 (0.87)**	**-0.61 (0.58)**	**-0.74 (0.85)**	**-0.32 (0.47)**
	-3.27 – 0.02, p = 0.033	-1.96 – 0.21, p = 0.117	-2.44 – 0.87, p = 0.194	-1.30 – 0.54, p = 0.244

3.2. HPI axis-related genes

For stress responses, it is essential to consider how the mRNA expression values of hormones, their binding proteins and receptors change relative to each other. A significant decrease of the ratio of *crf1:crh-bp* in the telencephalon and an increase in the hypothalamus was observed in stressed fish

compared with the control fish ($p = 0.021$, Figure 2). Furthermore, the ratio of *crf2:crh-bp* was decreased in the telencephalon by stress application ($p = 0.029$, Figure 2).





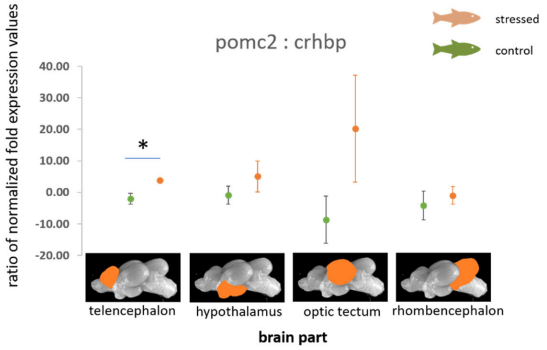


Figure 2. Ratios of the normalized fold expression of *crf1*, *crf2*, and *pomc2* relative to *crh-bp* in each of the 4 brain parts in the control fish and fish 30 min after the air exposure, mean ± SEM; n = 4 per treatment, significance according to the Mann-Whitney U tests, $p < 0.05$.

In addition, the ratios of the normalized fold expression of *pomc2* relative to *crh-bp* were found to be increased in the telencephalon of stressed fish compared with the control fish ($p = 0.034$, Figure 2). The ratio of the normalized fold expression of *pomc1* to the *crf* receptor 2 was found to be significantly decreased ($p = 0.043$, Figure 3).

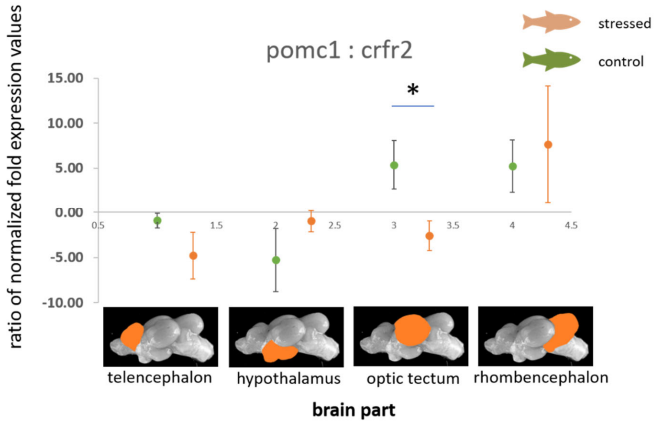


Figure 3. Ratios of the normalized fold expression of *pomc1* compared with the expression of *crfr2* in each of the 4 brain parts in the control fish and fish 30 min after the air exposure, mean ± SEM; n = 4 per treatment.

Kommentiert [PC1]: Achtung change figure

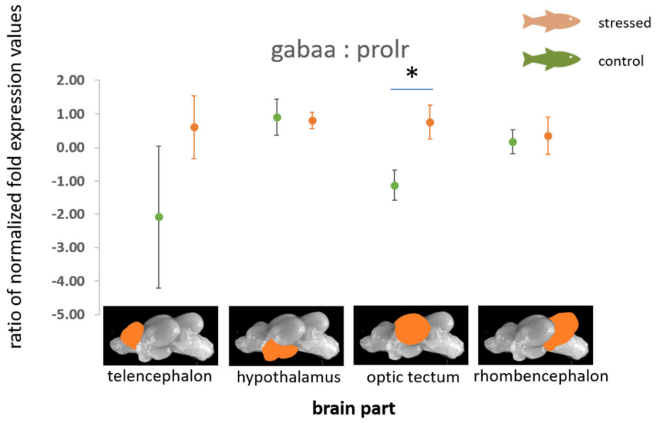
The PCA showed that the first two components of the PCA represented 74.5 % of the total variance in the HPI genes in the telencephalon, 74.2 % of the variance in the same genes in the hypothalamus, and 78.8 % and 60.6 % of the variance in these genes in the optic tectum and the rhombencephalon (Figure S2 in the supplement). A high cos2 value for *crf-r2* was observed in all four brain parts which indicates a good representation of this variable on the principal component. In these cases, the variables are positioned close to the circumference of the correlation circle in Figure S2. In contrast, a low cos2 indicates that the variable is not perfectly represented by the principle components. In this case the variable is close to the center of the circle which, for example, can be seen for *pomc2* in the telencephalon and rhombencephalon (Figure S2).

3.3. The gene expression patterns of the serotonergic genes, gabaa, isotocin precursor and the prolactin receptor

The ratios of the normalized fold expression of *gabaa* relative to *prolr* were found to be increased in the optic tectum of stressed fish compared with the control fish ($p = 0.021$, Figure 4). Furthermore, an increased probability of a reduction of *gabaa* expression in the optic tectum in relation to other genes that have been calculated in the present study (Table 1 and Figure S4). In addition, the ratio of the normalized fold expression of *serotr* relative to *gabaa* were found to be increased in the rhombencephalon of stressed fish compared with the control fish ($p = 0.021$, Figure 4), and increased probability of a reduction of *serotr* in the telencephalon in relation to other genes that have been included in the present study (Table 1 and Figure S4).

The PCA for the mRNA levels of the genes *5-ht-r*, *serotr*, *gabaa*, *isopre* and *prolr* in the telencephalon allowed the explanation of 72.2 % of the variance in the data set, whereas the same calculations in the hypothalamus revealed that 85.9 % of the variance are related to the selected genes (Figure S3 in the Supplement). Similarly, 83.6 % and 73.9 % of the variance was attributed to the selected genes in the

optic tectum and in the rhombencephalon, respectively (Figure S3 in the supplement). Consequently, an optimal set of genes was desired for a final PCA, for which *gabaa, crfr1, crfr2, mr, egr-1, 5-ht-r* and *c-fos* have been selected for each of the brain parts separately (Figure 5). The PCA for the telencephalon showed that 74.0 % of the variance in the data set could be explained when these genes have been selected as variables. Compared to this, the PCA for the hypothalamus showed that 89.5 % of the variance was covered by the selected genes. Similarly, the respective variance levels were as high as 93.5 % in the optic tectum and 86.1 % in the rhombencephalon.



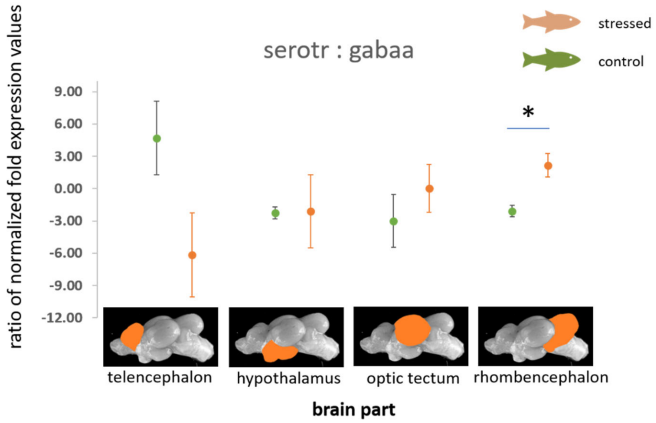


Figure 4. Ratios of the normalized fold expression of *gabaa* to *prolr* and the normalized fold expression of *serotr* to the expression of the *gabaa* gene in each of the 4 brain parts in the control fish and fish 30 min after the air exposure, mean ± SEM; n = 4 per treatment, significance according to the Mann-Whitney U tests, $p < 0.05$.

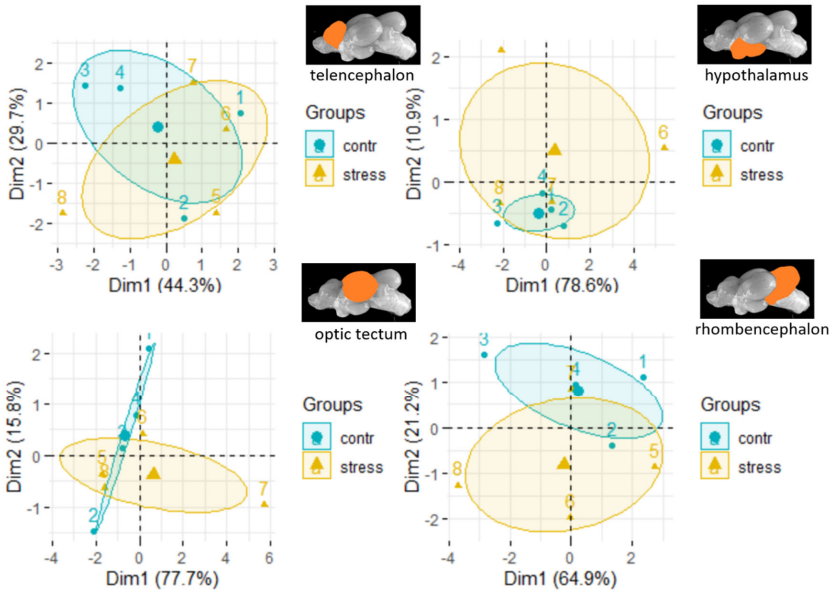


Figure 5. Results for the first two components (Dim1 and Dim2) of the PCA including confidence ellipses for the selected genes *gabaa, crfr1, crfr2, erg-1, mr, erg-1, 5-ht-r* and *c-fos* in each in the 4 brain parts of control fish and fish 30 min after the air exposure (the numbers in the brackets indicate the percentage of the variance in the data sets that is explained by each components, mean ± SEM; n = 4 per treatment.

4. Discussion

Fish reared in aquaculture systems are continuously exposed to different stimuli, some of them being able to reach a distress situation once they overcome the physiological limits of the animals. With a continuous growth of the fish farming industry, ways to define and quantify welfare become vital to generate recommendations for best practice and legislation adaptations. In this study, we aim to identify the diversity of gene expression profiles between the four brain parts (telencephalon, optic tectum, hypothalamus and rhombencephalon) of distressed common carp.

Reference genes are fundamental for the exact determination of changes in gene expression [52,55]. Usually these are chosen from previous studies but only a limited number has selected reference genes according to their stable expression pattern in distinct tissues [56,57]. Typical reference genes are related to maintenance of cell structures and metabolism. For fish brain no such detailed investigation of suitable reference genes has been performed before. The reason is that that even acute stress, for example in trout, can alter the gene expression of a number of genes involved in intracellular signalling and cytoskeleton changes [58]. Thus, the selection of housekeeping genes that typically have these functions in cells might be impaired. Other genes, such as *gapdh*, may show a high variability which makes them unsuitable as references genes [59,60]. Here, only three genes have been shown to be suitable as reference genes. Given the limited number of animals per treatment group and in order to confirm this hypothesis that for the fish brain region-specific reference genes

are needed, it is recommend to perform an additional study using a higher sample size to confirm the suitability of the analysed reference genes in brain regulation studies of fish.

3.1. Immediate Early Genes (IEGs)

Different IEGs have been investigated in the present study to identify brain activity in the different brain parts. The increased expression of *c-fos* in the telencephalon of distressed carp and the increased probability of a reduction of this gene in the optic tectum in relation to other genes (see Figure S4 for this) confirms that *c-fos* is not only a suitable indicator of brain activity in higher vertebrates and fish species such as zebrafish and goldfish [11,48,61], but also in carp. *In situ* hybridization has already shown that light avoidance leads to changes of the expression of *c-fos* in the medial zone of the dorsal telencephalon in adult zebrafish 30 min after the induction of neuronal activity [11]. Together with the present results from carp, it becomes clear that *c-fos* is capable of indicating changes in brain activity after exposure to acute stressors, in this case air exposure.

3.2. HPI axis-related Genes

That acute stress involves the *crf* system, e.g. preoptic area in the forebrain in fish, has already been reviewed in the past [61,62]. The fact that *crf* and *crh-bp* are widely distributed in the fish brain supports the assumption that the *crf* system has important functions even outside the cerebral system [63]. For example, changes in the *crf* system caused by stressors, including hypoxia, have also been observed in the caudal neurosecretory system and the heart of zebrafish [64,65]. *Crh-bp* is known to inhibit the *crf*-mediated activation of the *crf* receptors in a receptor subtype-specific fashion [66]. For this reason, the ratios of the normailzed fold expression of the *crf* genes in the present study have been compared to the level of *crh-bp*, and the application of an acute stressor appears to influence this ratio, proving the assumption that the *crh-bp* actions are receptor-specific. Previous investigations have indicated that *crh-bp* is a more potent inhibitor of the *crfr2* activation of than for *crfr1* in fish [66]. Similarly, the *crf*2 receptors in humans have been described as being coupled to the cAMP–PKA signaling pathways similar to *crfr2*, but they mediate effects opposite to those of *crfr1* receptors during arthritis [67]. This reflects that *crf1* and *crf*2 and also the two *crf* receptors have different functions which is probably also the case in fish. The differential effects of distress on the ratio of the normalized fold expression of the *crf* receptors compared with the fold expression of *crh-bp* in the present study support the assumption that both receptors have different functions in carp as well.

In the present study, also the ratio of the normalized fold expression of *pomc1* to *crfr2* is reduced in the optic tectum. The hypothalamic circuit includes two populations of neurons: one co-expressing orexigenic neuropeptides, such as neuropeptide Y, and the second one expressing pro-opiomelanocortin (*pomc*) and anorexigenic neuropeptides thus regulating feed intake in fish [68]. The funtions of pomc neurons in the optic tectum are less well described. Nevertheless, the resuts of the present study support the hypothesis that the optic tectum is more than a dominantly retinorecipient structure.

3.3. Genes of the serotonergic and the gaba-ergic pathway

A connection of prolactin release and GABA receptor signalling has previously been shown in the hypothalamus of rodents as well as in the pituitary in rodent and rainbow trout [47,49,50]. Surprisingly, the change of the ratio of the normalized fold expression of the GABA A receptor (*gabaa*) relative to the expression of the *prolr* was observed in the optic tectum, but the role of the *prolr* in this brain part remains unclear so far. The levels of GABA receptor mRNA expression together with the

expression of other important receptors, for example membrane receptors for serotonin and dopamine, affect memory loss in rats [69].

Serotonin transporter expression and activity which is required for returning serotonin to the presynaptic neuron where it can be degraded or retained for later re-use. In higher vertebrates, selective serotonin reuptake inhibitors can lead to increased GABA concentrations [70] which confirms an interaction of serotonin pathways and GABA levels. The present study on carp also indicated that the ratio of *serotr* to *gabaa* is influenced by acute distress. In trout, acute stress resulted in downregulation of a serotonin receptor subtype and *mr* in the telencephalon 4 h post stress compared with the levels 1 h post-stress which indicated that a negative feedback exists for these receptors that aims at downregulating the HPI axis after activation by stress [71]. More sampling time points would have been required in the present study to show the dynamics of the activation of similar feedback mechanisms in carp.

In rodents, maternal care increases the 5-ht turnover at the serotonin receptor increasing the activity of this receptor which leads to the activation of the expression of factors such as *egr-1* further downstream [72,73]. The PCA in the present study indicated that *egr-1* is a suitable gene to indicate changes in the brain regulation in carp as a result of exposure to distress.

5. Conclusions

This section is mandatory.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: "Variable correlation plot of the PCA for the IEG-related genes in each in the 4 brain parts", Figure S2: "Variable correlation plot of the PCA for the HPI axis-related genes in each in the 4 brain parts", Figure S3: "Variable correlation plot of the PCA for the serotonin- and *gabaa*-related genes in each in the 4 brain parts", Figure S4: "Posteriori probability plots of all genes in each in the 4 brain parts of control fish and fish 30 min after the air exposure", Table S1: "Primer pairs selected for the gene expression studies", Table S2: "Average expression stability (M value) of the potential reference genes",

Author Contributions: Conceptualization, methodology, investigation, funding acquisition, project administration, writing—original draft preparation, software, formal analysis and data curation, visualization, writing—review and editing: A.B. and C.P. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by the Bridge programme (Project No. 40B2-0_180864) supported by the Swiss National Science Fonds (SNSF) and the Innosuisse - Swiss Innovation Agency.

Conflicts of Interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

References

1. Moberg GP. Problems in defining stress and distress in animals. *J Am Vet Med Assoc*, 1207–1211.
2. Carstens, E., Moberg, G.P. Recognizing pain and distress in laboratory animals. *Ilar Journal* **2000**, 62–71.
3. Gornati, R.; Papis, E.; Rimoldi, S.; Terova, G.; Saroglia, M.; Bernardini, G. Rearing density influences the expression of stress-related genes in sea bass (Dicentrarchus labrax, L.). *Gene* **2004**, *341*, 111–118, doi:10.1016/j.gene.2004.06.020.
4. Liu, S.; Gao, G.; Palti, Y.; Cleveland, B.M.; Weber, G.M.; Rexroad, C.E. RNA-seq analysis of early hepatic response to handling and confinement stress in rainbow trout. *PLoS ONE* **2014**, *9*, e88492, doi:10.1371/journal.pone.0088492.
5. Sun, P.; Yin, F.; Tang, B. Effects of acute handling stress on expression of growth-related genes in Pampus argenteus. *J World Aquacult Soc* **2017**, *48*, 166–179, doi:10.1111/jwas.12323.

6. Samaras, A.; Espírito Santo, C.; Papandroulakis, N.; Mitrizakis, N.; Pavlidis, M.; Höglund, E.; Pelgrim, T.N.M.; Zethof, J.; Spanings, F.A.T.; Vindas, M.A.; et al. Allostatic load and stress physiology in European Seabass (Dicentrarchus labrax L.) and gilthead seabream (Sparus aurata L.). *Front. Endocrinol. (Lausanne)* **2018**, *9*, 451, doi:10.3389/fendo.2018.00451.
7. Vindas, M.A.; Gorissen, M.; Höglund, E.; Flik, G.; Tronci, V.; Damsgård, B.; Thörnqvist, P.-O.; Nilsen, T.O.; Winberg, S.; Øverli, Ø.; et al. How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. *J Exp Biol* **2017**, *220*, 1524–1532, doi:10.1242/jeb.153213.
8. Aronson, L.R. Forebrain function in teleost fishes. *Transactions of the New York Academy of Sciences* **1967**, *29*, 390–396, doi:10.1111/j.2164-0947.1967.tb02269.x.
9. Trotha, J.W.; Vernier, P.; Bally-Cuif, L. Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. *Eur J Neurosci* **2014**, *40*, 3302–3315, doi:10.1111/ejn.12692.
10. Lal, P.; Tanabe, H.; Suster, M.L.; Ailani, D.; Kotani, Y.; Muto, A.; Itoh, M.; Iwasaki, M.; Wada, H.; Yaksi, E.; et al. Identification of a neuronal population in the telencephalon essential for fear conditioning in zebrafish. *BMC Biol.* **2018**, *16*, 45, doi:10.1186/s12915-018-0502-y.
11. Lau, B.Y.B.; Mathur, P.; Gould, G.G.; Guo, S. Identification of a brain center whose activity discriminates a choice behavior in zebrafish. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 2581–2586, doi:10.1073/pnas.1018275108.
12. Paton, J.J.; Belova, M.A.; Morrison, S.E.; Salzman, C.D. The primate amygdala represents the positive and negative value of visual stimuli during learning. *Nature* **2006**, *439*, 865–870, doi:10.1038/nature04490.
13. Murray, E.A. The amygdala, reward and emotion. *Trends in Cognitive Sciences* **2007**, *11*, 489–497, doi:10.1016/j.tics.2007.08.013.
14. Morrison, S.E.; Salzman, C.D. Re-valuing the amygdala. *Curr. Opin. Neurobiol.* **2010**, *20*, 221–230, doi:10.1016/j.conb.2010.02.007.
15. Johansen, J.P.; Cain, C.K.; Ostroff, L.E.; LeDoux, J.E. Molecular mechanisms of fear learning and memory. *Cell* **2011**, *147*, 509–524, doi:10.1016/j.cell.2011.10.009.
16. Ingle, D.J. Optic Tectum. In *Comparative Neuroscience and Neurobiology*; Irwin, L.N., Ed.; Birkhäuser Boston: Boston, MA, 1988; pp 100–101, ISBN 978-1-4899-6776-3.
17. Springer, A.D., Easter, S.S., Agranoff, B.W. The role of the optic tectum in various visually mediated behaviors of goldfish. *Brain Research* **1977**, 393–404.
18. Roeser, T., Baier, H. Visuomotor behaviors in larval zebrafish after GFP-guided laser ablation of the optic tectum. *The Journal of Neuroscience* **2003**, 3726–3734.
19. Williams, K.W.; Elmquist, J.K. From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. *Nat Neurosci* **2012**, *15*, 1350–1355, doi:10.1038/nn.3217.
20. Moens, C.B.; Prince, V.E. Constructing the hindbrain: insights from the zebrafish. *Dev. Dyn.* **2002**, *224*, 1–17, doi:10.1002/dvdy.10086.
21. Durán, E.; Ocaña, F.M.; Martín-Monzón, I.; Rodríguez, F.; Salas, C. Cerebellum and spatial cognition in goldfish. *Behav. Brain Res.* **2014**, *259*, 1–8, doi:10.1016/j.bbr.2013.10.039.
22. Duman, R.S.; Adams, D.H.; Simen, B.B. Transcription factors as modulators of stress responsivity. In *Handbook of stress and the brain*; Steckler, T., Ed.; Elsevier: Amsterdam, 2005; pp 679–698, ISBN 9780444511737.
23. Ashlin, T.G.; Blunsom, N.J.; Ghosh, M.; Cockcroft, S.; Rihel, J. Pitpnc1a regulates zebrafish sleep and wake behavior through modulation of insulin-like growth factor signaling. *Cell Reports* **2018**, *24*, 1389–1396, doi:10.1016/j.celrep.2018.07.012.

24. Chatterjee, D.; Tran, S.; Shams, S.; Gerlai, R. A simple method for immunohistochemical staining of zebrafish brain sections for c-fos protein expression. *Zebrafish* **2015**, *12*, 414–420, doi:10.1089/zeb.2015.1147.
25. Salierno, J.D., Snyder, N.S., Murphy, A.Z., Poli, M., Hall, S., Baden, D., Kane, A.S. Harmful algal bloom toxins alter c-fos protein expression in the brain of killifish, Fundulus heteroclitus. *Aquatic Toxicology* **2006**, *78*, 350–357, doi:10.1016/j.aquatox.2006.04.010.
26. Kent, M.; Bell, A.M. Changes in behavior and brain immediate early gene expression in male threespined sticklebacks as they become fathers. *Horm. Behav.* **2018**, *97*, 102–111, doi:10.1016/j.yhbeh.2017.11.002.
27. Randlett, O.; Wee, C.L.; Naumann, E.A.; Nnaemeka, O.; Schoppik, D.; Fitzgerald, J.E.; Portugues, R.; Lacoste, A.M.B.; Riegler, C.; Engert, F.; et al. Whole-brain activity mapping onto a zebrafish brain atlas. *Nat. Methods* **2015**, *12*, 1039–1046.
28. Boukhelifa, M., Parast,M.M., Valtschanoff, J.G., LaMantia, A.S., Meeker, R.B., Otey, C.A. A role for the cytoskeleton-associated protein palladin in neurite outgrowth. *Molecular Biology of the Cell* **2001**, 2721–2729.
29. Jia, Z.; Chen, L.; Ge, Y.; Li, S.; Peng, W.; Li, C.; Zhang, Y.; Hu, X.; Zhou, Z.; Shi, L.; et al. Genetic mapping of Koi herpesvirus resistance (KHVR) in Mirror carp (Cyprinus carpio) revealed genes and molecular mechanisms of disease resistance. *Aquaculture* **2020**, *519*, 734850, doi:10.1016/j.aquaculture.2019.734850.
30. Artelt, N.; Ludwig, T.A.; Rogge, H.; Kavvadas, P.; Siegerist, F.; Blumenthal, A.; van den Brandt, J.; Otey, C.A.; Bang, M.-L.; Amann, K.; et al. The role of palladin in podocytes. *J Am Soc Nephrol* **2018**, *29*, 1662–1678, doi:10.1681/ASN.2017091039.
31. Flik, G.; Klaren, P.H.M.; van den Burg, E.H.; Metz, J.R.; Huising, M.O. CRF and stress in fish. *Gen. Comp. Endocrinol.* **2006**, *146*, 36–44, doi:10.1016/j.ygcen.2005.11.005.
32. Conde-Sieira, M.; Chivite, M.; Míguez, J.M.; Soengas, J.L. Stress effects on the mechanisms regulating appetite in teleost fish. *Front. Endocrinol. (Lausanne)* **2018**, *9*, 631, doi:10.3389/fendo.2018.00631.
33. Doyon, C.; Trudeau, V.L.; Moon, T.W. Stress elevates corticotropin-releasing factor (CRF) and CRF-binding protein mRNA levels in rainbow trout (Oncorhynchus mykiss). *J. Endocrinol.* **2005**, *186*, 123–130, doi:10.1677/joe.1.06142.
34. Huising, MO., Metz, J. R., van Schooten, C., Taverne-Thiele, A. J., Hermse, T., Verburg-van Kemenade, B. M., Flik, G. Structural characterisation of a cyprinid (Cyprinus carpio L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. *J. Mol. Endocrinol.* **2004**, *32*, 627–648, doi:10.1677/jme.0.0320627.
35. Seasholtz, A.F., Valverde, R.A., Denver, R.J. Corticotropin-releasing hormone-binding protein: biochemistry and function from fishes to mammals. *Journal of Endocrinology* **2002**, 89–97.
36. Sturm, A.; Bury, N.; Dengreville, L.; Fagart, J.; Flouriot, G.; Rafestin-Oblin, M.E.; Prunet, P. 11-deoxycorticosterone is a potent agonist of the rainbow trout (Oncorhynchus mykiss) mineralocorticoid receptor. *Endocrinology* **2005**, *146*, 47–55, doi:10.1210/en.2004-0128.
37. Stolte, E.H.; Mazon, A.F. de; Leon-Koosterziel, K.M.; Jesiak, M.; Bury, N.R.; Sturm, A.; Savelkoul, H.F.J.; van Kemenade, B.M.L.V.; Flik, G. Corticosteroid receptors involved in stress regulation in common carp, Cyprinus carpio. *J. Endocrinol.* **2008**, *198*, 403–417, doi:10.1677/JOE-08-0100.
38. Gesto, M.; López-Patiño, M.A.; Hernández, J.; Soengas, J.L.; Míguez, J.M. Gradation of the stress response in rainbow trout exposed to stressors of different severity: The role of brain serotonergic and dopaminergic systems. *J. Neuroendocrinol.* **2015**, *27*, 131–141, doi:10.1111/jne.12248.
39. Pantoja, C.; Hoagland, A.; Carroll, E.; Schoppik, D.; Isacoff, E.Y. Measuring behavioral individuality in the acoustic startle behavior in zebrafish. *Bio Protoc* **2017**, *7*, e2200, doi:10.21769/BioProtoc.2200.

40. Ohlsen, R.I.; Pilowsky, L.S. The place of partial agonism in psychiatry: recent developments. *Journal of Psychopharmacology* **2005**, *19*, 408–413, doi:10.1177/0269881105053308.
41. Kumar, S.; Porcu, P.; Werner, D.F.; Matthews, D.B.; Diaz-Granados, J.L.; Helfand, R.S.; Morrow, A.L. The role of GABA(A) receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology (Berl)* **2009**, *205*, 529–564, doi:10.1007/s00213-009-1562-z.
42. Abreu, M.S.; Giacomini, A.C.V.; Gusso, D.; Rosa, J.G.S.; Koakoski, G.; Kalichak, F.; Idalêncio, R.; Oliveira, T.A.; Barcellos, H.H.A.; Bonan, C.D.; et al. Acute exposure to waterborne psychoactive drugs attract zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **2016**, *179*, 37–43, doi:10.1016/j.cbpc.2015.08.009.
43. Sokołowska, E.; Gozdowska, M.; Kulczykowska, E. Nonapeptides arginine aasotocin and isotocin in fishes: Advantage of bioactive molecules measurement. *Front. Mar. Sci.* **2020**, *7*, doi:10.3389/fmars.2020.00610.
44. Manzon, L.A. The Role of Prolactin in Fish Osmoregulation: A Review. *Gen. Comp. Endocrinol.* **2002**, *125*, 291–310, doi:10.1006/gcen.2001.7746.
45. Power, D.M. Developmental ontogeny of prolactin and its receptor in fish. *Gen. Comp. Endocrinol.* **2005**, *142*, 25–33, doi:10.1016/j.ygcen.2004.10.003.
46. Patil, M.J.; Henry, M.A.; Akopian, A.N. Prolactin receptor in regulation of neuronal excitability and channels. *Channels (Austin)* **2014**, *8*, 193–202, doi:10.4161/chan.28946.
47. Grattan, D.R. 60 years of neuroendocrinology: The hypothalamo-prolactin axis. *Journal of Endocrinology* **2015**, *226*, T101-T122, doi:10.1530/JOE-15-0213.
48. Fujikawa, Y.; Kozono, K.; Esaka, M.; Iijima, N.; Nagamatsu, Y.; Yoshida, M.; Uematsu, K. Molecular cloning and effect of c-fos mRNA on pharmacological stimuli in the goldfish brain. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* **2006**, *1*, 253–259, doi:10.1016/j.cbd.2005.12.005.
49. Sarkar, D.K. Evidence for prolactin feedback actions on hypothalamic oxytocin, vasoactive intestinal peptide and dopamine secretion. *Neuroendocrinology* **1989**, *49*, 520–524, doi:10.1159/000125161.
50. Prunet, P.; Gonnard, J.F.; Paboeuf, G. GABA-ergic control of prolactin release in rainbow trout (Oncorhynchus mykiss) pituitaries in vitro. *Fish Physiol. Biochem.* **1993**, *11*, 131–137, doi:10.1007/BF00004559.
51. Vandescompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **2002**, research0034.1.
52. Taylor, S.C.; Nadeau, K.; Abbasi, M.; Lachance, C.; Nguyen, M.; Fenrich, J. The ultimate qPCR experiment: Producing publication quality, reproducible data the first time. *Trends Biotechnol.* **2019**, *37*, 761–774, doi:10.1016/j.tibtech.2018.12.002.
53. Zhu, A.; Srivastava, A.; Ibrahim, J.G.; Patro, R.; Love, M.I. Nonparametric expression analysis using inferential replicate counts. *Nucleic acids research* **2019**, *47*, e105, doi:10.1093/nar/gkz622.
54. Bürkner, P.-C. Advanced Bayesian multilevel modeling with the R package brms. *The R Journal* **2018**, 1–15.
55. Remans, T.; Keunen, E.; Bex, G.J.; Smeets, K.; Vangronsveld, J.; Cuypers, A. Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy. *Plant Cell* **2014**, *26*, 3829–3837, doi:10.1105/tpc.114.130641.
56. Rojas-Hernandez, N.; Véliz, D.; Vega-Retter, C. Selection of suitable reference genes for gene expression analysis in gills and liver of fish under field pollution conditions. *Sci. Rep.* **2019**, *9*, 3459, doi:10.1038/s41598-019-40196-3.

57. Øvergård, A.-C., Nerland, A.H., Patel, S. Evaluation of potential reference genes for real time RT-PCR studies in Atlantic halibut (Hippoglossus Hippoglossus L.); during development, in tissues of healthy and NNV-injected fish, and in anterior kidney leucocytes. *BMC Molecular Biology* **2010**, 36–51.
58. Krasnov, A.; Koskinen, H.; Pehkonen, P.; Rexroad, C.E.; Afanasyev, S.; Mölsä, H. Gene expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics* **2005**, *6*, 3, doi:10.1186/1471-2164-6-3.
59. Kocmarek, A.L., Ferguson, M.M, Danzmann, R.G. Differential gene expression in small and large rainbow trout derived from two seasonal spawning groups. *BMC Genomics* **2014**, 57–76.
60. Barber, R.D.; Harmer, D.W.; Coleman, R.A.; Clark, B.J. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiological Genomics* **2005**, *21*, 389–395, doi:10.1152/physiolgenomics.00025.2005.
61. Pavlidis, M.; Theodoridi, A.; Tsalafouta, A. Neuroendocrine regulation of the stress response in adult zebrafish, Danio rerio. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2015**, *60*, 121–131, doi:10.1016/j.pnpbp.2015.02.014.
62. Bernier, N.J. The corticotropin-releasing factor system as a mediator of the appetite-suppressing effects of stress in fish. *Gen. Comp. Endocrinol.* **2006**, *146*, 45–55, doi:10.1016/j.ygcen.2005.11.016.
63. Alderman, S.L.; Bernier, N.J. Localization of corticotropin-releasing factor, urotensin I, and CRF-binding protein gene expression in the brain of the zebrafish,Danio rerio. *J. Comp. Neurol.* **2007**, *502*, 783–793, doi:10.1002/cne.21332.
64. Bernier, N.J.; Flik, G.; Klaren, P.H.M. Chapter 6: Regulation and contribution of the corticotropic, melanotropic and thyrotropic axes to the stress response in fishes. *Fish Physiology : Fish Neuroendocrinology*; Academic Press, 2009; pp 235–311, ISBN 1546-5098.
65. Williams, T.A.; Bergstrome, J.C.; Scott, J.; Bernier, N.J. CRF and urocortin 3 protect the heart from hypoxia/reoxygenation-induced apoptosis in zebrafish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **2017**, *313*, R91-R100, doi:10.1152/ajpregu.00045.2017.
66. Manuel, R.; Metz, J.R.; Flik, G.; Vale, W.W.; Huising, M.O. Corticotropin-releasing factor-binding protein (CRF-BP) inhibits CRF- and urotensin-I-mediated activation of CRF receptor-1 and -2 in common carp. *Gen. Comp. Endocrinol.* **2014**, *202*, 69–75, doi:10.1016/j.ygcen.2014.04.010.
67. Fu, Y.; Neugebauer, V. Differential mechanisms of CRF1 and CRF2 receptor functions in the amygdala in pain-related synaptic facilitation and behavior. *J. Neurosci.* **2008**, *28*, 3861–3876, doi:10.1523/JNEUROSCI.0227-08.2008.
68. Delgado, M.J.; Cerdá-Reverter, J.M.; Soengas, J.L. Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: Involvement in the control of food intake. *Front. Neurosci.* **2017**, *11*, 354, doi:10.3389/fnins.2017.00354.
69. Tellez, R.; Gómez-Viquez, L.; Liy-Salmeron, G.; Meneses, A. GABA, glutamate, dopamine and serotonin transporters expression on forgetting. *Neurobiol. Learn. Mem.* **2012**, *98*, 66–77, doi:10.1016/j.nlm.2012.05.001.
70. Sanacora, G.; Mason, G.F.; Rothman, D.L.; Krystal, J.H. Increased occipital cortex GABA concentrations in depressed patients after therapy with selective serotonin reuptake inhibitors. *American Journal of Psychiatry* **2002**, *159*, 663–665, doi:10.1176/appi.ajp.159.4.663.
71. Moltesen, M.; Laursen, D.C.; Thörnqvist, P.-O.; Andersson, M.Å.; Winberg, S.; Höglund, E. Effects of acute and chronic stress on telencephalic neurochemistry and gene expression in rainbow trout (Oncorhynchus mykiss). *J. Exp. Biol.* **2016**, *219*, 3907–3914, doi:10.1242/jeb.139857.

564 72. Meaney, M.J.; Szyf, M. Maternal care as a model for experience-dependent chromatin plasticity? *Trends*
565 *Neurosci.* **2005**, *28*, 456–463, doi:10.1016/j.tins.2005.07.006.
566 73. Meaney, M.J.; Diorio, J.; Francis, D.; Weaver, S.; Yau, J.; Chapman, K.; Seckl, J.R. Postnatal handling
567 increases the expression of cAMP-inducible transcription factors in the rat hippocampus: The effects of
568 thyroid hormones and serotonin. *J. Neurosci.* **2000**, *20*, 3926, doi:10.1523/JNEUROSCI.20-10-03926.2000.
569
570 **Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional
571 affiliations.
572

573